

Production of protease isozymes by *Aphanomyces cochlioides* and *Aphanomyces euteiches*[☆]

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Abstract

The production of protease activity by the sugarbeet pathogen *Aphanomyces cochlioides*, the legume pathogen *A. euteiches*, and the fish pathogen *Saprolegnia parasitica* was examined. Protease activity was readily detected in supernatants of water cultures of each organism using autoclaved host tissue as a nutrient source. Most of the protease isozymes extracted from sugarbeet and pea seedlings infected with *A. cochlioides* and *A. euteiches*, respectively, co-migrated with enzymes produced by the pathogens in culture. In inoculated sugarbeet seedlings, the protease activities were detected prior to or concomitant with the onset of disease symptoms and the activities were capable of digesting protein extracted from sugarbeet hypocotyls. Use of class-specific inhibitors indicated that a portion of the protease activity was of the trypsin-class. Trypsin-like isozymes that possessed a relatively fast electrophoretic migration were detected in the *A. cochlioides*, *A. euteiches*, and *S. parasitica* protease complements, whereas the remaining isozymes were not affected by any of the inhibitors tested. Proteinaceous trypsin inhibitors from the legumes lima bean (*Phaseolus lunatus*) and soybean (*Glycine max*) inhibited the trypsin-like isozymes from *A. cochlioides*, but not *A. euteiches*, whereas low molecular weight, synthetic trypsin inhibitors inhibited these isozymes from both pathogen sources. The potential role of protease inhibition in determining host range in phytopathogenic *Aphanomyces* species is discussed.

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Keywords: *Beta vulgaris* L.; *Aphanomyces*; *Saprolegnia*; Protease; Virulence

1. Introduction

Root rot and seedling diseases induced by the genus *Aphanomyces* cause considerable losses to legume and sugarbeet growers where wet, warm field conditions occur [16]. Reduced yields are observed in alfalfa, pea and snap bean production due to infection by *A. euteiches* [13] and reduced stands, yield, and quality of sugarbeet results from infection by *A. cochlioides* [24]. Both of these organisms can persist in fields that have not been planted to a host crop for 10 or more years. Quantitative, heritable resistance to *Aphanomyces* has been described for alfalfa [10], pea [7,14], and sugarbeet [6], although sugarbeet seedlings of resistant

varieties remain highly susceptible to disease. For this reason, sugarbeet seed planted into soils with a history of black root seedling disease continues to require treatment with the anti-oomycotic hymexazol (Tachigaren™) for optimal seedling establishment [5,18].

Historically, studies of extracellular enzyme production by root rotting pathogens have focused on cell wall-degrading enzymes [2,23]. This followed from the ability of pectinases and cellulases to induce tissue maceration when they were infiltrated into healthy tissue. Less attention has been paid to the involvement of protease activity in the virulence of phytopathogens, even though such activities potentially would be able to participate in cell wall weakening [17,23], in the inactivation of host-derived defense proteins such as pathogenesis-related (PR) proteins [15], and in the disruption of cellular functions necessary to mount defense against pathogen attack [21].

Species of *Aphanomyces* that attack fish (e.g. *A. invadans*) and crayfish (e.g. *A. astaci*) have been shown to produce abundant extracellular protease, an activity putatively involved in the degradation of collagen during

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the penetration of host epidermis [9]. Molecular genetic research on the proteases produced by the crayfish pathogen *Aphanomyces astaci* has led to the cloning of genes for two serine proteases produced by this organism [4]. *Saprolegnia parasitica* is an oomycete pathogen of fish that has been taxonomically aligned with the *Aphanomyces* and has been selected as a model organism for members of the Saprolegniaceae [12]. In the present work, protease activity produced by *A. cochlioides* and *A. euteiches* was examined and compared to that from *S. parasitica*. For the three oomycetes tested, abundant protease activity was present in culture supernatants; additionally, high protease levels were detected in host seedlings infected with the two phytopathogens. Differential inhibition of a trypsin-like protease produced by *A. cochlioides* and *A. euteiches* by inhibitors of leguminous sources is discussed in terms of the respective host range of these pathogens.

2. Methods

2.1. Cultures, reagents and inhibitors

Single zoospore isolates of *A. cochlioides* (19-1z), *A. euteiches* (MN174), and *S. parasitica* ATCC200015 were maintained in oatmeal broth or on cornmeal agar (CMA) at 22 °C in the dark. Electrophoresis and protease assay reagents along with proteinaceous inhibitors, azocasein substrate, and trypsin standards were purchased from Sigma Chemical (St Louis, MO USA). A protease inhibitor test kit was purchased from Roche Applied Sciences (Indianapolis, IN USA). Enzyme electrophoresis was performed on a Mini-Protean II vertical slab system (BioRad Laboratories, Hercules, CA USA).

2.2. Growth conditions, seedling inoculations, and extract preparation

Transverse slices of mature sugarbeet roots (*Beta vulgaris* cv ACH9369), whole roots and hypocotyls of 6-week-old pea plants (*Pisum sativum* cv 'Wando'), and chopped pieces of smelt fish (*Osmerus mordax*) were autoclaved in excess distilled water. Single sugarbeet slices (for *A. cochlioides*), multiple root and hypocotyl pieces (for *A. euteiches* culture), and smelt pieces (for *S. parasitica*) were transferred to 20 ml sterile, double-distilled water (ddH₂O) in Petri dishes followed by inoculation with agar plugs containing the respective pathogens. Cultures were incubated at 22 °C in the dark. Protease activity in the culture supernatant was examined weekly during initial time-course characterization and at 14 days post-initiation for all other analyses.

At 2 weeks post-planting, seedlings of sugarbeet and pea in containers (Stuewe and Sons Inc., Corvallis OR USA) were inoculated with zoospores of *A. cochlioides* and *A. euteiches*, respectively. Zoospores were induced from mycelium as previously described [14]. Five millilitres of

water containing 5×10^4 zoospores were applied to each container. Plants were incubated in a greenhouse with an average temperature of 24 °C and a 16 h photoperiod. Pea seedlings were harvested when water-soaked symptoms first appeared. Sugarbeet seedlings were harvested at 0, 24, 48, 72, and 96 h post-inoculation. Seedlings from each container were combined and weighed. Extracts were prepared by the grinding the seedlings from each container in 0.1 M Tris-HCl pH 6.8, 1.0 mM Na₂EDTA, and 10% glycerol at a rate of 3 ml per gram of fresh weight tissue. Following low speed centrifugation, the supernatants were stored at -20 °C until used.

2.3. Analysis of total protease activity

Supernatants from pathogen cultures were prepared by filtration through Miracloth™ (Calbiochem) and were stored at -20 °C. Protease assays were initiated by mixing 120 µl of supernatant with 60 µl of azocasein suspension (2% w/v in 50 mM Tris-HCl pH 8.0) modified from the method of Sarath et al. [20]. Mixtures were incubated at 37 °C and subsets of the samples were removed at various time points to analyze the extent of proteolysis. Samples removed from incubation were mixed with three volumes of 15% trichloroacetic acid (TCA) and placed on ice. After the samples had incubated in TCA in ice for 0.5 h, the precipitate (undigested azocasein) was removed by centrifugation at 14,000g for 5 min. Recovered supernatants were mixed with an equal volume of 1 M NaOH and analyzed spectrophotometrically at 440 nm. For the analysis of protease inhibition, inhibitors were used at the concentrations noted in Table 1, with the exception of inhibitors from lima bean and soybean which were included in gels and solution assays at 50 µg/ml. At least three independent cultures for each organism were analyzed where each data point represents the mean of triplicate protease assays run in parallel. Standard statistical and regression analysis tools within the Microsoft™ Excel spreadsheet software were used to examine data.

The effect of pH on protease activity was determined by adjusting the supernatant to 0.1 M sodium acetate (for pH 5.2), sodium citrate (for pH 6.0), and Trizma (for pH's 7, 7.5, 8.0, 8.8, and 9.5) prior to initiation of the digestion assay. Comparison of protease inhibition in supernatants of *A. cochlioides* and *A. euteiches* was done by diluting the *A. cochlioides* preparation with water to normalize the total protein content by the Bradford assay (BioRad). Percent inhibition was estimated as a decrease in absorbance at 440 nm after 24 h in comparisons between reactions containing no inhibitor with those containing protease inhibitors of different sources and chemistries.

2.4. Electrophoretic analysis of protease isozymes

Discontinuous polyacrylamide gel electrophoresis of the enzymes in the presence of sodium dodecylsulfate

Table 1
Inhibition of protease activity secreted by *A. cochlioides* and *A. euteiches* by class-specific inhibitors^a

Inhibitor	Concentration ^b	Class	Trypsin (11 mg/ml)	<i>A. cochlioides</i> supernatant		<i>A. euteiches</i> supernatant	
				Absorbance ^c	% Inh.	Absorbance ^c	% Inh.
(none)			1.193(0.006)	0.838(0.064)	(0)	0.52(0.041)	(0)
E-64	10 µg/ml	Cysteine	1.092(0.040)	0.823(0.039)	2	0.345(0.032)	34
Antipain	50 µg/ml	Serine/cysteine	0.215(0.009)	0.565(0.015)	33	0.495(0.020)	5
Bestatin	40 µg/ml	Metallo-	1.132(0.043)	0.823(0.088)	2	0.429(0.158)	17
Leupeptin	5 µg/ml	Serine/cysteine	1.031(0.066)	0.599(0.033)	29	0.444(0.052)	15
Pepstatin	0.7 µg/ml	Aspartic	1.092(0.073)	0.786(0.046)	6	0.483(0.035)	7
Phosph.	330 µg/ml	Metallo-	1.169(0.055)	0.751(0.070)	10	0.501(0.047)	4
AEBSF	1 mg/ml	Serine	0.659(0.093)	0.499(0.170)	41	0.279(0.027)	46
EDTA	0.5 mg/ml	Metallo-	1.13(0.029)	0.758(0.053)	10	0.434(0.046)	17
Aprotinin	2 µg/ml	Serine	1.032(0.19)	0.648(0.150)	23	0.425(0.034)	18

^a Azocasein was digested at 37 °C for 24 h with or without inhibitor.

^b Final concentration of inhibitor in the digestion.

^c Absorbance value at 440 nm and standard deviation (in parentheses). Values where no inhibitor was used in the supernatants of *A. cochlioides* (A.c.) and *A. euteiches* (A.e.) represent the controls from which the percent inhibition (% inh.) is obtained.

(SDS-PAGE) separated the various protease activities [20]. Gels were cast between glass plates separated by 1.5 mm spacers and contained 10% acrylamide, 0.27% bisacrylamide, 0.38 M Tris–HCl pH 8.8, 0.1% SDS, 0.1% gelatin, 0.05% ammonium persulfate and 0.001% TEMED. The stacking portion of the gels contained 4% acrylamide, 0.1% bisacrylamide, 0.13 M Tris–HCl pH 6.8, 0.1% SDS, 0.05% ammonium persulfate and 0.001% TEMED. In-gel assay of protease inhibition was done either by co-polymerization of the inhibitor in the gel or by addition of the inhibitor to the incubation buffer after electrophoresis [20]. Extracts from cultures were mixed with a 4× concentrated loading dye to achieve a final concentration of 15 mM Tris–HCl pH 6.8, 2% SDS, 10% sucrose, and 0.02% bromophenol blue. Reducing agents were omitted from the system for these tests. To test sensitivity of protease isozymes to heat and to reducing agents, samples were mixed with loading dye and incubated in either a boiling water bath for 10 min without added β-mercaptoethanol or at 37 °C for 10 min in the presence of 0.72 M β-mercaptoethanol prior to loading the gel. Electrophoresis was performed at 21 V/cm at room temperature until the bromophenol blue tracking dye reached the bottom of the gel. Gels were removed from the plates, incubated in 2% Triton X-100 for 1 h at room temperature, and incubated over night in 2% Triton X-100, 0.1 M Tris–HCl pH 8.0, 1 mM CaCl₂ at 37 °C. Gels were stained with Coomassie brilliant blue solution and destained in 10% methanol/10% acetic acid. Finally, gels were dried under vacuum onto Whatmann 3 MM paper for archiving and were scanned with a digital color flatbed scanner (UMAXTM Vista S-12).

In additional experiments, beet protein was used as substrate for zymogram gel analysis. For these tests, 2-week-old sugarbeet hypocotyls were ground in 0.1 M Tris–HCl pH 6.8, 1.0 mM Na₂EDTA, 14 mM β-mercaptoethanol and 10% glycerol followed by clarification of the extract by centrifugation. Total protein was precipitated by the addition of 8 ml of cold acetone and 1 ml of 100%

trichloroacetic acid per ml of clarified extract. Samples were incubated for 60 min at –20 °C before the precipitate was collected by centrifugation at 18,000g for 15 min at 4 °C. The pellet was rinsed with cold acetone, dried in vacuo, and dissolved in water for addition to the acrylamide gel mix. Precipitated protein from 15–20 hypocotyls was mixed with 10 ml of acrylamide solution for gel casting. Following the separation of *A. cochlioides* isozymes on these gels, zymograms were developed as described above.

3. Results

3.1. The phytopathogenic *Aphanomyces* produce abundant protease activity

Supernatants of cultures in which *A. cochlioides* (A.c.) and *A. euteiches* (A.e.) were grown on autoclaved sugarbeet root slices and pea root sections, respectively, were found to contain protease activity in digestion of azocasein (Figs. 1A and 4A). The pH optimum of the activity was found to be 8.0 (Fig. 1A); therefore all subsequent solution and gel assays were performed at this pH. Sugarbeet seedlings infected with A.c. possessed protease activity capable of digesting ~4× more azocasein after a 24 h period than that found in extracts of healthy seedlings (Fig. 2A). The effects of protease inhibitors indicated that a serine protease class comprised much of the activity in A.e. and A.c. (Table 1). The evaluation included compounds for the inhibition of serine, aspartyl, cysteine, and metalloproteases. With this information, similar tests comparing the inhibition of protease activity in culture supernatants of A.c. showed the increased effectiveness of the synthetic serine protease inhibitor AEBSF over that of the proteinaceous trypsin inhibitor from lima bean (LBTI; Fig. 4A).

The supernatants of cultured A.c., A.e., and S.p. contain proteases capable of digesting gelatin in activity gels (Fig. 1) and A.c. proteases are capable of digesting total

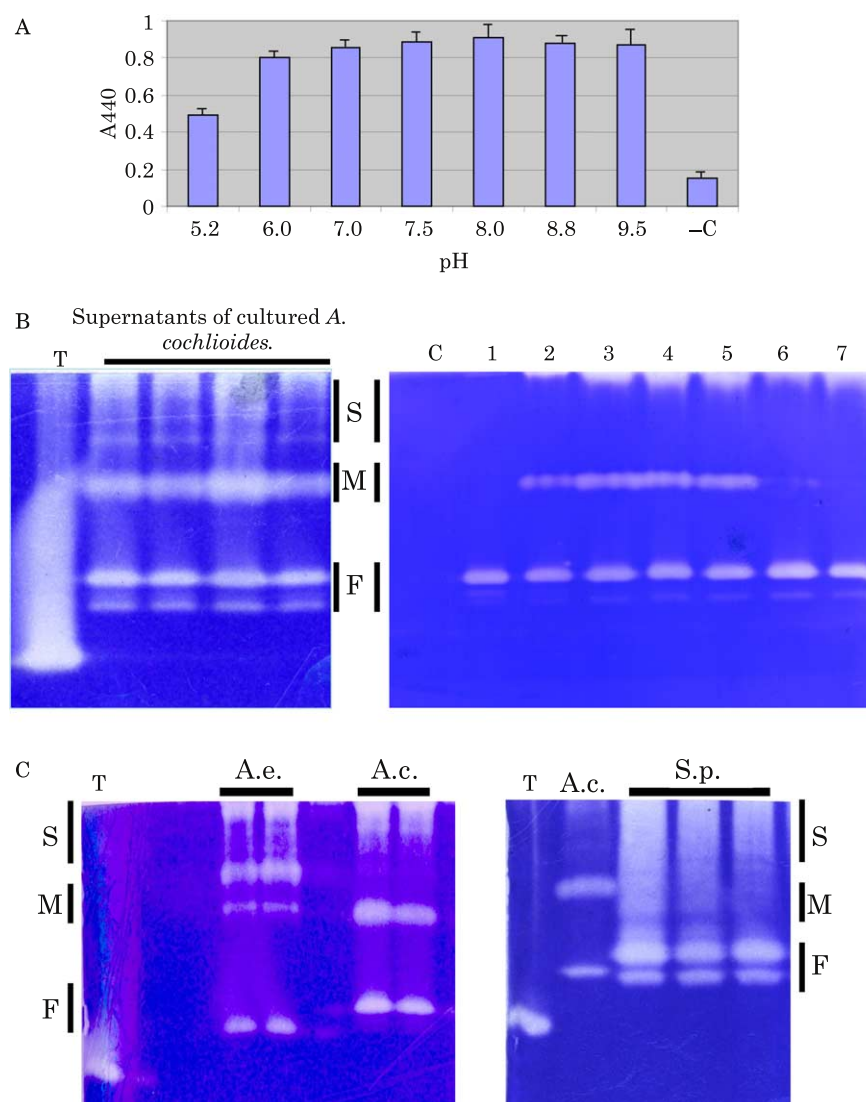


Fig. 1. Dependence of total protease activity secreted by *A. cochlioides* on pH and protease isozymes produced by *A. cochlioides*, *A. euteiches* and *S. parasitica*. Total protease activity measured as digestion of azocasein by preparations from A.c. was substantial over a broad pH range (A). Error bars denote standard deviation. Separation of *A. cochlioides* isozymes in gelatin-containing PAGs (B) to generate bands of slow (S), medium (M), and fast (F) relative migration. Left panel shows isozymes produced from four independent field isolates of *A. cochlioides*. Right panel shows isozymes produced by one isolate of *A. cochlioides* 1–7 weeks after culture initiation as denoted above the lanes. Lane C contains supernatant from uninoculated beet-slice culture after 7 days of incubation. Co-electrophoresis of commercial trypsin (T) provided a size and digestion standard for B and C. The left panel in C illustrates relative differences in protease migration between cultures of *A. cochlioides* (A.c.) and *A. euteiches* (A.e.) and the right panel shows a comparison between proteases from A.c. and *S. parasitica* (S.p.). For A, B (left panel) and C, supernatants used were harvested after 3 weeks of incubation.

protein extracted from sugarbeet hypocotyls (Fig. 3). A time-course assay of isozyme production by A.c. indicated that most of the activities are produced between 2 and 6 weeks after culture initiation (Fig. 1B). A total of 7–8 isozymes present in the A.c. supernatants and 5–6 isozymes present in A.e. supernatants were separable on gelatin zymograms gels using this approach. For convenience, the isozymes are referred to as slow (S), medium (M), and fast (F) with regards to their relative migration in the gel (Fig. 1). Slight variation in the intensity of some of the isozymes between experiments in the study, particularly in the slow migrating proteases, appears to reflect sensitivity of these isozymes to freeze–thaw cycles.

Although similar in their distribution, the protease isozymes produced by the three oomycete pathogens did not co-migrate in this gel system, which includes SDS at 0.1%. Presence of SDS in the gel and loading buffer would impart a net negative charge to the isozymes, but in the absence of a reducing agent such as β -mercaptoethanol, the isozymes will not migrate proportionally to their molecular mass. This is revealed by the anomalous migration of trypsin (MW=23,800) relative to the molecular size standards (not shown).

Evaluation of extracts from sugarbeet and pea seedlings infected with A.c. and A.e., respectively, suggested that many of the protease isozymes are produced in planta

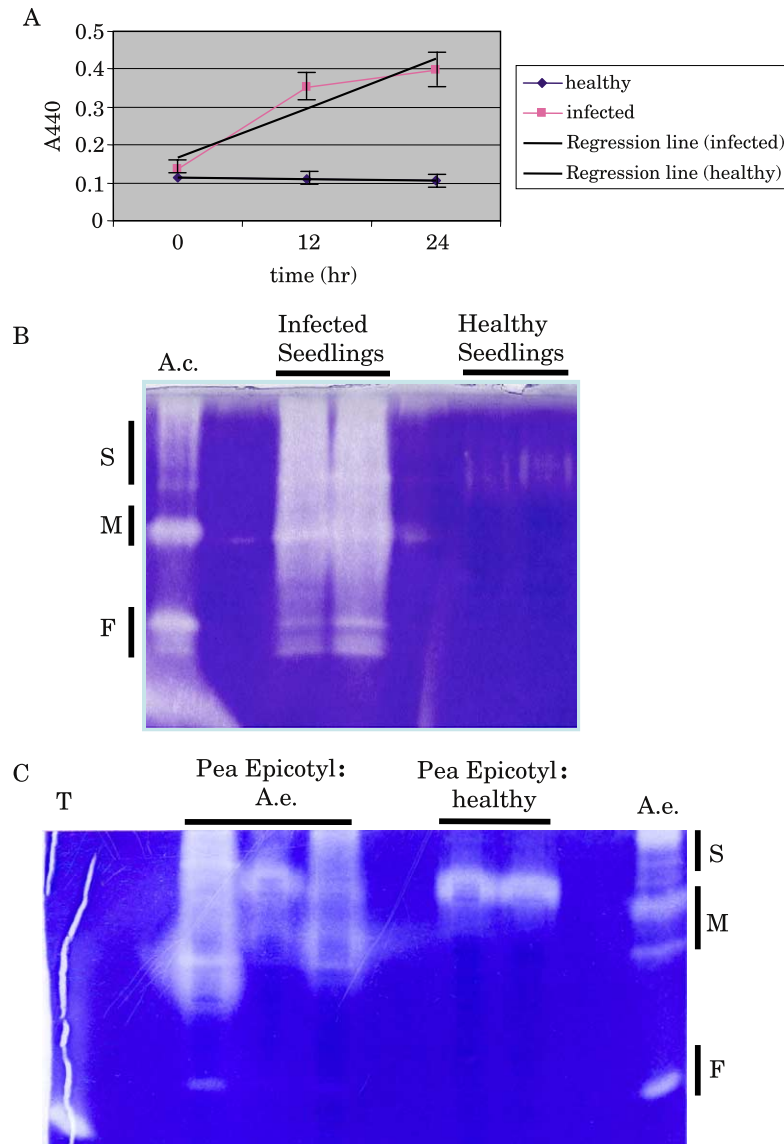


Fig. 2. Comparison of total and separated protease activity in healthy sugarbeet and pea seedlings with those infected with *A. cochlioides* and *A. euteiches*, respectively. Plant extracts were prepared 5 days after inoculation with pathogen zoospores. Protease activity increased approximately fourfold in extracts of sugarbeet seedlings infected with *A. cochlioides* as compared to healthy seedlings after 24 h of digestion of azocasein (A). In B and C, the complement of isozymes observed in culture filtrates of *A. cochlioides* (A.c.) and *A. euteiches* (A.e.) also are observed in infected seedlings of sugarbeet (B) and pea (C), respectively. The relative migration of trypsin (T) serves as a control.

(Figs. 2B and 2C). Minimal protease activity was detected in healthy sugarbeet hypocotyls and pea epicotyls, facilitating the comparison of the protease isozyme complement with that from extracts of infected seedlings. Many, but not all, of the protease isozymes produced in pure culture co-migrated with those produced in infected tissue. The protease activities were detected without concentrating the extracts, indicating that the enzymes were abundantly produced in liquid culture and host plant tissues. Time course analysis indicated that protease activity was detected by zymography within 3 days post-inoculation, just before, or concomitant with, the onset of early disease symptoms (Fig. 3A).

A test of the sensitivity of the isozymes to heat and a reducing agent indicated that the faster migrating isozymes,

but not the slower migration forms, were sensitive to 0.72 M β -mercaptoethanol. Conversely, the slower migrating isozymes were sensitive to boiling, whereas the faster migrating isozymes were little affected by this treatment (not shown).

3.2. Trypsin-like protease activities are present in culture supernatants

Use of inhibitors specific for the serine protease class indicated that a portion of the activity produced in culture was comprised of trypsin-like activities (Table 1, Figs. 4 and 5). Acrylamide activity gels revealed that the fastest migrating activities were inhibited by inhibitors specific for

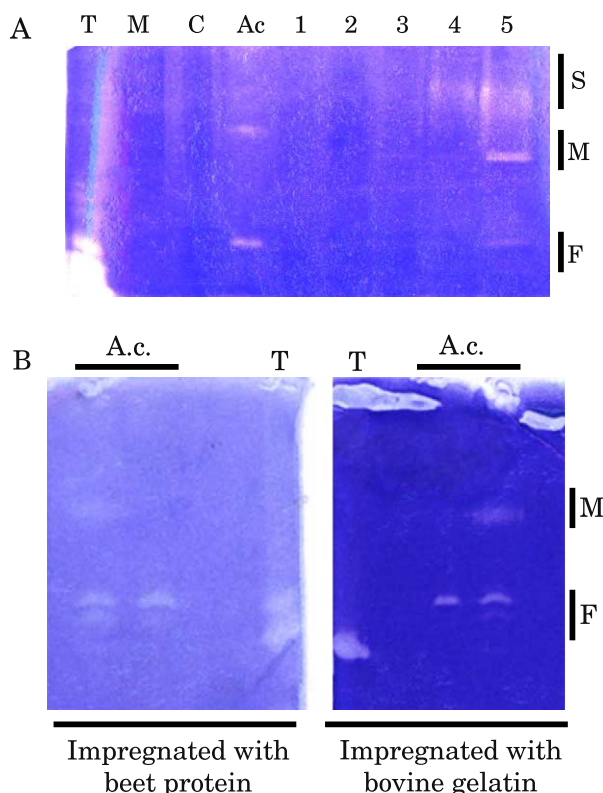


Fig. 3. Time course of appearance of *A. cochlioides* protease in inoculated sugarbeet seedlings and digestion of sugarbeet protein by protease activities. Zymogram (A) showing protease produced by *A. cochlioides* in culture (Ac) and seedlings harvested at 0, 24, 48, 72, and 96 h (lanes 1–5) post-inoculation with *A. cochlioides* zoospores. In B, impregnation of zymogram gels with protein from sugarbeet hypocotyls (left side) was compared to gels containing gelatin (right side) for sensitivity to *A. cochlioides* protease. Trypsin (T) was co-electrophoresed as a control and S, M, and F denote the slow, medium, and fast migrating isozymes, respectively, along the side of the panels. The 'M' lane in A is a molecular weight standard.

trypsin-like enzymes, whereas the slower migrating isozymes were unaffected by this treatment. Both protein and small, synthetic inhibitors blocked the trypsin activities of A.c. in this system (Figs. 4 and 5).

Solution assays indicated that inclusion of a synthetic inhibitor of serine protease, Pefabloc™ [4-(2-aminoethyl)-benzenesulfonyl-fluoride, hydrochloride (AEBSF)], reduced the extent of proteolysis after 24 h by a factor of 2.8 (Fig. 4A). A proteinaceous trypsin inhibitor from lima bean (LBTI) inhibited the extent of digestion by a factor of 1.7. By comparison, an insignificant reduction in the rate of protease digestion of azocasein was observed when leupeptin was added at the recommended concentration (not shown).

3.3. The trypsin-like activities are differentially inhibited

The sensitivities of the trypsin-like isozymes of A.e. and A.c. were compared. AEBSF inhibited the fast migrating proteases of both *Aphanomyces* species (Fig. 5, lower panel). Interestingly, LBTI (Fig. 5, middle panel and Fig. 4B)

and soybean trypsin inhibitor (not shown) inhibited the fast-migrating isozymes from A.c., but not from A.e.. The slower migrating proteases were not significantly affected by treatment with trypsin-class inhibitors.

4. Discussion

Investigation into the biochemical basis for the production of macerated tissue in infections by root rotting phytopathogens has focused on the production of carbohydrases [2,23]. The production of protease as a component of virulence has received attention recently, but it has been studied as a major virulence factor only in the oomycetes that infect animals. Thus, penetration of the crayfish cuticle by *Aphanomyces astaci* [4] and of human epidermis by *Pythium insidiosum* [19] is proposed to involve digestion by protease.

In the present work, the phytopathogens *A. cochlioides* and *A. euteiches* were shown to produce abundant protease activity in culture and in infected plants. The co-migration of some isozymes produced by *A. cochlioides* and *A. euteiches* in infected plant tissue with those produced in pure culture supports the contention that these isozymes originate from the pathogen. Differences between other isozymes produced in culture filtrates and those produced in infected plants may reflect the production of novel proteases by the plant in response to infection, the production of novel proteases by the pathogen in response to plant invasion, or the post-translational modification of isozymes produced in culture upon the interaction with host plant. Generation of antisera specific for individual isozymes purified from culture supernatants would prove useful in resolving this issue.

The analysis of proteases secreted in culture by *S. parasitica*, an oomycete closely related to the *Aphanomyces*, revealed activities with similar, but distinct, electrophoretic migration in activity gels. The results suggest that abundant protease secretion is a hallmark of the Saprolegniales, whose lineage diverges from the phytopathogenic *Phytophthora* and *Pythium* species [12]. Consistent with this, *Phytophthora infestans* is known to secrete low amounts of protease activity [17], but protease inhibitors are readily detected in cultures of this organism [12].

The bulk of the fast migrating protease activity in culture filtrates of *A. cochlioides* and *A. euteiches* belonged to the trypsin class based on the effects of specific inhibitors. The trypsin-like protease activity produced by *Aphanomyces* is not required for saprophytic growth, since inclusion of inhibitors into agar media had no noticeable effect on radial growth rate of the mycelium (not shown). Of particular interest was the observation of near complete inhibition of the fast migrating proteases from *A. cochlioides* by the trypsin inhibitors from lima bean and soybean. These same inhibitors were less

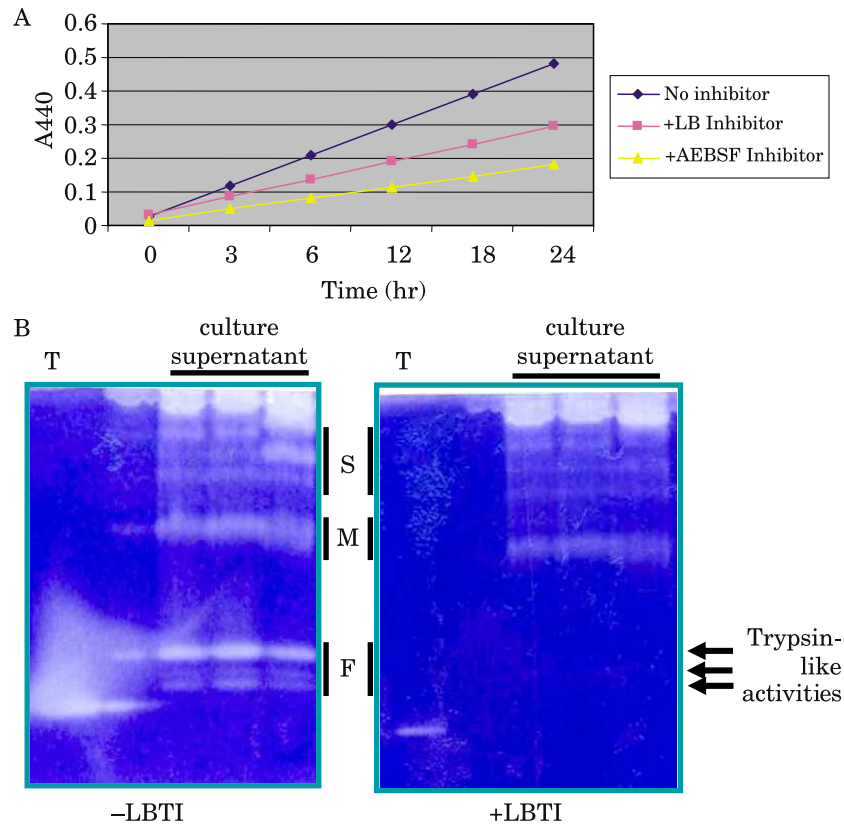


Fig. 4. Relative inhibitory activity of a proteinaceous trypsin inhibitor from lima bean (LB) and the synthetic inhibitor AEBSF and inhibition of specific protease isozymes from cultured *A.c.* In A, the regression line indicates the more potent inhibitory potential of AEBSF over that of LB inhibitor in a time course of the digestion of azocasein. Inhibition with lima bean trypsin inhibitor (LBTI) of protease isozymes from *A. cochlioides* supernatants separated by SDS-PAGE reveals that the fast migrating isozymes are trypsin-class proteases (B). The relative migration of trypsin (T) serves as an electrophoretic control.

effective against the analogous proteases from *A. euteiches*. An intriguing possibility is that protease activity is a virulence determinant in the phytopathogenic *Aphanomyces* species, whereby a variant protease has evolved in *A. euteiches* (a legume pathogen) to evade inhibition by a proteinaceous inhibitor from a legume source. This would be analogous to the diversifying selection that has been found to characterize fungal polygalacturonases and their inhibitors encoded by plant hosts [8]. Future cloning and analysis of protease gene homologues from these two fungi would provide valuable information towards answering this question.

The remaining proteases of slower migration were not appreciably inhibited by trypsin class inhibitors or those targeted to the remaining protease classes. It is possible that some artifact of the gel system prohibits access of the slower migrating isozymes by the inhibitors. Alternatively, these isozymes may represent novel protease classes against which the inhibitors tested are ineffective. Treatment of the samples with heat or a reducing agent prior to electrophoresis indicated that the fast and slow migrating proteases differed in their stability with the fast migrating species possessing greater heat resistance and the slower migrating species possessing greater resistance to β -mercaptoethanol. The variation in isozyme size,

class, and chemistry likely will prove useful in purification schemes for these isozymes as well as in the taxonomic characterization of *Aphanomyces* species and other Oomycetes.

Previous work has documented the production of carbohydrase activities by plant [3,11] and invertebrate [1] pathogenic species of *Aphanomyces*. These activities are thought to participate in softening of the tissue during invasion of the plant tissue by the pathogen, a role that protease secretion may also play. The detection of elevated protease activity in pea tissue by *A. euteiches* has been noted previously, but was proposed to be involved in the induced resistance to the pathogen both in the presence and absence of the arbuscular mycorrhiza *Glomus mosseae* [22]. The discovery here of pathogen-derived protease activity in sugarbeet and pea seedling tissue infected with *Aphanomyces* alone suggests that this activity may have a role in tissue degradation. Alternatively, the secreted proteases may act to destroy non-structural proteins such as PR- or other cellular proteins, thereby enabling unobstructed invasion by the pathogen. Detection of the activities in sugarbeet seedlings infected with *A. cochlioides* before the onset of symptoms supports the proposition that they are indeed involved in one or both of these processes. The use of proteinase inhibitors will be used in future investigations to

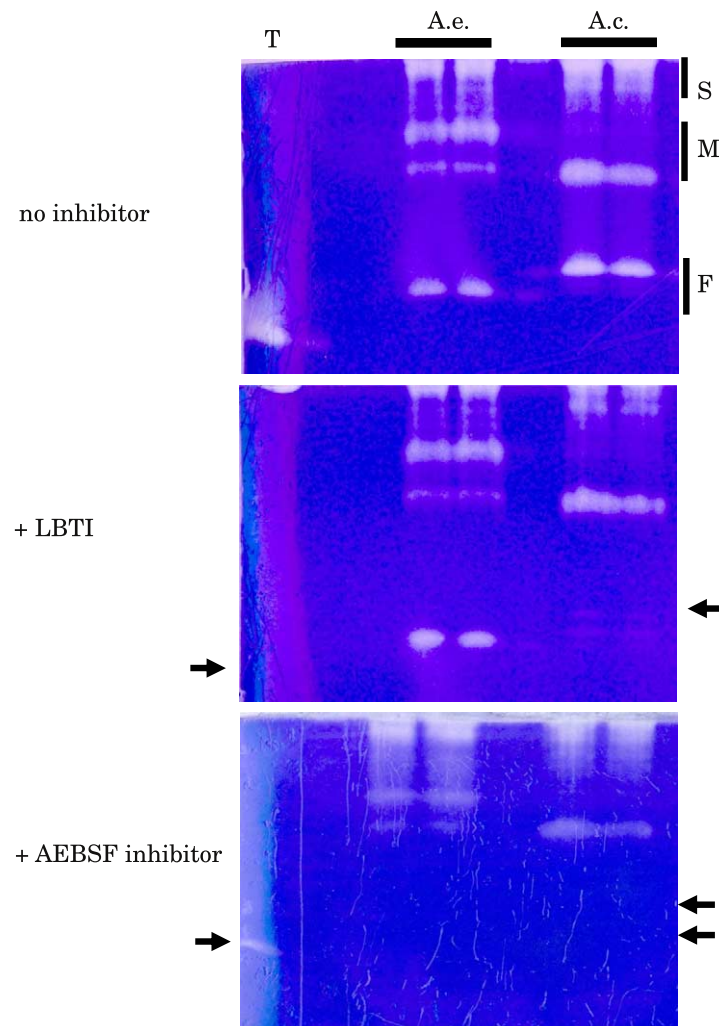


Fig. 5. Comparison of the inhibition of isozymes produced in culture by *A. cochlioides* (A.c.) and *A. euteiches* (A.e.) using lima bean trypsin inhibitor (LBTI) and AEBSF. The top panel shows a full isozyme complement after SDS-PAGE when no inhibitor is present. In the presence of LBTI (middle panel) the fast-migrating proteases from *A. cochlioides* are inhibited, whereas those from *A. euteiches* are not. In the lower panel, all fast migrating protease isozymes are inhibited by the synthetic inhibitor AEBSF. Arrows denote the position of inhibited isozymes (right side of panel) and commercial trypsin (T lane; left side of panel).

determine the role of protease secretion in the infection of sugarbeet seedlings by *A. cochlioides*. Purification of individual protease isozymes from *A. cochlioides* will be instrumental in their biochemical characterization and will aid indirectly in the cloning the genes encoding these enzymes.

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